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## **INTRODUCTION**

The Crk protein was originally identified as the oncogene fusion product of the CT10 chicken retrovirus (v-Crk) (1). Cellular homologues of v-Crk, include c-Crk, which encodes two alternatively spliced proteins (c-CrkI and c-CrkII), and c-CrkL. Crk proteins are composed of one Src homology 2 (SH2), and one or two Src homology 3 (SH3) domains (2). The Crk adaptor proteins (CrkI, CrkII and CrkL) play an important role during cellular signalling (of processes such as migration, invasion, & adhesion) by mediating the formation of protein complexes. Previous work in our lab has shown that the over-expression of Crk proteins promotes an invasive phenotype, regardless of upstream signalling implicating a role for Crk proteins in cancer (3, 4). Moreover, Crk proteins are over-expressed in multiple types of cancer, including lung carcinoma, glioblastoma, and breast cancer (5, 6, 7). Although many studies have focused on the over-expression of Crk being important for many cellular processes, their significance in inherently motile cancer cells still remains elusive. Furthermore, much of what has been proposed about Crk proteins in epithelial cells is based upon in vitro over-expression studies or the use of dominant interfering mutants of CrkI/II or CrkL. These approaches, while informative, are limited by the challenges intrinsic to over-expression systems and these in vitro assays do not truly reflect the complexity of an in vivo response. Thus the goal of our study was to examine if Crk adaptor proteins are truly responsible for mediating signals required for migration and invasion by validating how loss of Crk would affect these processes. Through the use of short interfering RNA (siRNA) silencing techniques and in vivo model systems, I will establish experimental models to formally test the hypothesis that *Crk proteins play an important role in cellular migration, invasion and metastasis, through their ability to recruit specific protein-protein complexes*. Our study on Crk adaptor proteins has the potential to identify some of the molecular events, which can occur during metastasis. Furthermore, since Crk proteins are over-expressed in human breast cancer, I will establish mouse models to test the hypothesis that *over-expression of Crk can promote breast tumorigenesis*. This may provide information, which could be used to develop effective treatments for breast cancer.

## **BODY:**

### **(1) Role of Crk in cellular migration and invasion in various types of cancer**

Several studies have suggested that Crk mediates cellular migration and invasion downstream of various stimuli. *I tested the hypothesis that Crk functions as a central adaptor protein that integrates upstream signals for cell migration and invasion by examining how the loss of Crk expression affects these processes in highly invasive cancer cell lines*. Using RNAi, I targeted CrkI, CrkII and CrkL in highly malignant breast cancer cell lines (MDA-435s and MDA-231) and found that the loss of Crk was associated with a decrease in cellular invasion and migration (7). In addition, migration was significantly decreased even after stimulation with HGF, a known stimulant of cellular migration (7). These results demonstrate that Crk adaptor proteins play an important role in the promotion of signals for cell migration and invasion in human breast cancer cell lines irrespective of the upstream regulatory events that promote the invasive response. These results are now published in Molecular Cancer Research (7).

I extended these studies by analyzing Crk dependent migration and invasion (using a siRNA-mediated approach) on a panel of breast cancer cell lines available in the lab (ie. BT-474, SKBR3, BT-20, BT-549, Hs578T) in order to gain a better understanding of the precise role of Crk adaptor proteins in breast cancer. Certain breast cancer cell lines (BT-474, SKBR3) were unable to either migrate or invade, suggesting that they are not highly aggressive. Although Crk proteins are present in these cell lines, the ability of Crk to inhibit these processes was unable to be tested by this method. Indeed, it has been recently shown by other groups that these cell lines are not highly invasive or motile (8). More interestingly, gene expression profiling on a wide panel of human breast cancer cell lines have distinguished breast cancer cell lines into two distinct classes – luminal and basal, results similar to those obtained from human breast cancer patients (8, 9). The cell lines which were poorly migratory and invasive (BT-474, SKBR3) fell into the luminal category, which is associated with good prognosis (8, 9).

Other cell lines tested (BT-20, BT-549) were considered to be of a basal phenotype, which is associated with poor prognosis and decreased survival in human patients (8, 9, 10). When Crk siRNA was transfected into basal cell lines, inhibition of cellular migration and invasion was quite significant. For instance, invasion was inhibited anywhere from 30-90% depending on the cell line examined. These data demonstrate that Crk adaptor proteins play an important role in integrating signals for migration and invasion

of highly malignant cancer cell lines. Furthermore, this information, coupled with the known biological properties of Crk, identifies a potential role for Crk in metastasis. This has important implications as we have shown elevated levels of Crk are observed in human breast cancer (7). Studies by Tanaka and colleagues have examined the role of Crk in ovarian cancer and have found that Crk is important for cellular migration and invasion through RNAi-based studies as well (11). They have found that loss of Crk inhibits tumor formation and metastatic growth. However, their metastatic assays fail to recapitulate all aspects of the metastatic cascade, and thus, the precise role of Crk during metastasis has yet to be revealed. Furthermore, the metastatic ability of ovarian cancer is different from breast cancer, thus the precise role of Crk in breast cancer metastasis still needs to be addressed.

## **(2) Role of Crk adaptor proteins in metastasis**

Since Crk proteins are over-expressed in various types of cancer, including breast cancer, and Crk appears to play a key role in processes required for metastasis, *I hypothesize that Crk proteins play a critical role in the metastatic spread of human breast tumors.* To test this hypothesis, I will utilize both cell-based studies and animal models to better understand the role of Crk in the metastatic cascade. To examine the role of Crk in metastasis, I will inject MDA-231 breast cancer variants expressing stable Crk, CrkL or triple knockdown (CrkI/II, CrkL) shRNA into nude mice and determine the number of metastases formed from “knockdown” cells versus control cells. The parental cell line, MDA-231 TR has the ability to form lung metastases when injected into the tail vein or the mammary fat pad (12, 13). The variant cell line, MDA-231 1833-TR has a higher propensity to form bone metastases compared to the parental cell line when injected into the left cardiac ventricle (12). These cell lines are tagged with a dual GFP-firefly luciferase tag (TR) which allows for the monitoring of metastatic development over time. If I am successful in preventing or lessening the formation of metastases in orthotopic transplant models, I can conclude that Crk plays an important role in the metastatic cascade of breast cancer.

I currently have stable clonal cell lines that have constitutive and specific knockdown of CrkI/II (pSuper-CrkI/II) or CrkL (LMP-CrkL) in both parental and bone variant MDA231 cell lines (Appendix 1-4). For the pSuper-CrkI/II cell lines, several in vitro assays were performed to confirm that the stable knockdown cells act similar to transient knockdown. For instance, the ability of these Crk shRNA constructs to decrease migration and invasion was tested in vitro using the boyden chambers described in Chapter 1. Stable knockdown of CrkI/II lead to significant decreases in cellular migration and invasion. Several optimization steps have been carried out to accurately quantitate migration and invasion data. The previous quantification technique used in the lab (as seen in (7)) was quantifying migration and invasion through the average pixel count of the images. However, several problems have been encountered recently using this technique. Firstly, the filters on the transwells have been skewing the data. Secondly, the stable cell lines result in a different cell shape, such that they are large and spread out compared to the small, fibroblast control cells. By basing the analysis on average area of the image, it over-represents the number of knockdown cells actually present. Thus, we have devised new software to count the number of cells individually instead of the over-all area. Although bugs of the program are still being worked out, it looks promising.

In addition to the migration and invasion assays, preliminary results suggest that loss of CrkI/II does not alter cellular proliferation through the use of cell doubling assays, however Alamar Blue assays will be performed to gain quantitative data to confirm this finding. Finally, stable knockdown of CrkI/II did not significantly inhibit growth in soft agar. Data for the parental MDA231TR cell line is found in Appendix 1, whereas data for MDA231 1833TR cell line is found in Appendix 2.

In addition to the stable clones expressing CrkI/II shRNA, I also obtained clones inhibiting CrkL (LMP-CrkL). Stable knockdown of CrkL was tested for their ability to inhibit tumor growth in nude mice. No significant changes were noted in MDA231TR parental cell lines (Appendix 3), however, knockdown of CrkL in one clone in the MDA231 1833TR bone variant cell line significantly inhibited growth (Appendix 4). Other clones will be tested to determine whether CrkL truly inhibits cellular growth or not. This is important as the role of CrkL in breast cancer progression has not yet been elucidated. Like the CrkI/II knockdown cell lines, the CrkL cell lines were tested in vitro to ensure that stable clones which have specific knockdown of CrkL also inhibits migration and invasion of these cell lines. Through the use of in vitro

assays, it was determined that stable knockdown of CrkL did not significantly inhibit proliferation or growth in soft agar, nor cellular migration and invasion in both MDA231TR (Appendix 3) and MDA231 1833TR cell lines (Appendix 4). Interestingly, when these cell lines were transfected with CrkI/II siRNA, it led to enhanced inhibition of cellular migration and invasion; greater than CrkI/II or CrkL knockdown alone. Thus, I created stable cell lines that contained the two shRNA targeting CrkI/II and CrkL leading to a triple knockdown.

Cellular proliferation assays need to be repeated using Alamar Blue assays, however, preliminary results suggest that loss of all three Crk proteins did not significantly affect cell proliferation but did inhibit cellular migration and invasion in both MDA231TR and MDA231 1833TR cell lines (Appendix 5&6). For the MDA231TR cell lines, the cells were grown to inject into mice for the metastasis assays, however, they lost their triple knockdown in culture, suggesting that the cells could not tolerate loss of all 3 Crk proteins in culture for long periods of time. New stable knockdown cells are being generated for the MDA231TR cell lines and they will be injected right away into the mice to prevent deselection of knockdown from occurring in cell culture.

For the MDA231 1833TR cell lines, the knockdown was sustainable and the metastasis assays should be finished within the next 2 months. Cardiac injections will be performed and the mice will be monitored every 4 days to examine the luciferase activity within the mice (which represents the formation of metastases). Bone x-rays will be taken at later time points and the number and size of metastatic lesions will be compared between the control and knockdown cell lines.

I anticipate that ablation of all 3 Crk proteins will act to prevent or lessen the formation of bone and lung metastases in these models. If this is true, these experiments will formally establish that Crk plays an important role in the metastatic cascade of breast cancer. No prior data exists suggesting that Crk regulates these processes for breast cancer metastasis. To confirm that Crk proteins are indeed modulating the metastatic phenotype, rescue experiments will be performed in which cDNA expressing CrkI, CrkII or CrkL will be stably expressed into these MDA-231 cell variants to determine if the phenotype is recapitulated. This has important implications, as most studies that examine the Crk protein (which includes the isoforms CrkI and CrkII) never specify which of these two proteins are required for the migratory or invasive phenotype.

### **(3) To examine Crk dependent signaling pathways involved in cancer cell migration and invasion**

Although I have demonstrated that Crk adaptor proteins act as key integrators for cellular migration and invasion, the exact mechanism by which Crk mediates such pathways has not been shown. Previous studies by Tanaka and colleagues have shown that loss of Crk leads to decreased Rac activation, which is required for cellular migration, however no further studies have been published detailing how the loss of Crk affects migration and invasion of inherently motile cancer cells (11). Originally, we had proposed to look at the role of Crk downstream from integrins, CD44 and various growth factors. However, the scope of that chapter was quite wide, thus we decided to adopt an unbiased approach to study how Crk regulates tumor progression. In this study, I will utilize microarray analysis to gain insight into the Crk dependent molecular mechanisms underlying migration and invasion of MDA-231 breast cancer cells. Over-expression of Crk can lead to the activation of various transcriptional pathways (AP-1, SRE-1) and Crk is a known activator of JNK and Rac, which can induce transcription (14, 15). Microarray analysis will allow me to identify the extent to which Crk is required for the regulation of genes involved in cellular migration and invasion and hence, determine the specific signals mediated by Crk. Breast cancer cell lines whose cellular migration and invasion are Crk dependent (i.e. MDA-231TR) will be transiently transfected with non-targeting siRNA, Crk siRNA, CrkL siRNA, or combinations of the two (Crk, CrkL) and subjected to array analysis. A major advantage of this system, which distinguishes it from previous knockout approaches, is that the cells do not have to grow for long periods without Crk, and will thus, not have the opportunity to accumulate mutations or adjust expression levels of genes that might compensate for the loss of Crk. Multiple siRNAs will be used in this experiment to control for potential off-targeting effects caused by siRNA duplexes (20). Finally, the activation of downstream signals of Crk will also be examined (ie. Rac, JNK) to determine if loss of Crk impairs their activation. By carrying out these experiments, it may help us link the novel target genes Crk

affects to a specific pathway Crk is known to activate (ie. Rac, JNK, Rap). The goal of the research is to determine a specific Crk signature, which is a group of genes regulated by Crk proteins.

The Crk “signatures” derived from this study could then be compared to human breast cancer array data. For instance, a division of Dr. Morag Park’s lab, the Breast Cancer Functional Genomics Group (BCFGG), have generated gene expression profiles from laser capture microdissected tumor and matched-normal breast epithelium. For each patient whose tumor has been analyzed by microarray analysis, a 10-year follow-up history has been documented in a database developed by our lab. By comparing Crk-mediated profile to the BCFGG data on the same microarray platform, as well as to publicly available human data sets (ie. Van’t Veer, Sorlie/Perou), we may be able to correlate Crk dependent signatures to specific breast cancer subtypes and to prognosis.

Since microarray analysis provides a large amount of experimental data, data sets will be filtered to the top over-expressed and under-expressed genes (with a fold change above 2) and the expression level of a subset of genes will be confirmed by quantitative real-time PCR and Western blot analysis. Furthermore, I am currently transfecting the MDA231TR cell line with vectors that over-express each Crk protein (CrkI, CrkII and CrkL) as well as making cell lines that have triple over-expression (CrkI/CrkII, CrkL). We would like to carry out microarray analysis on these cell lines to compare the gene profiles to those obtained from the knockdown experiment. Any genes that are altered in both data sets could then be validated as Crk specific. The role of these genes in tumor progression will be further characterized using traditional biochemical assays, such as RNAi, immunoprecipitation, Western blot analysis, dominant negative mutants, and immunofluorescence. Thus, this study has the potential to elucidate novel mechanisms by which Crk mediates essential components of tumor progression. The results from this study may also give insight into the mechanisms of metastasis we are studying in Chapter 2.

Several different siRNA duplexes targeting CrkI/II or CrkL were ordered and optimized in MDA-231TR cells. A primary microarray experiment was carried out using a small subset of samples (Mock, Scramble, CrkI/II, CrkL, CrkI/II/CrkL) to confirm that this experiment would show differences between the siRNAs. Preliminary array results are shown in Appendix 7. RNA has been isolated from MDA-231TR cell lines expressing these different duplexes and knockdown has been confirmed through Western blot analysis. Microarray experiments and analysis are ongoing and results should be obtained within the next 2 months. I have also optimized Rac/Rho/Cdc42 assays in order to test if loss of Crk inhibits Rac activation because confirming the downstream effectors affected by loss of Crk may determine the exact mechanism by which Crk affects transcription of certain target genes.

#### **(4) Role of Crk adaptor proteins in the development of breast cancer using transgenic mice**

To examine *the importance of Crk adaptor proteins in tumor progression*, I will use a transgenic mouse model, in which Crk proteins are over-expressed in the mammary epithelium. I have created constructs for CrkI, CrkII and CrkL, which allows the over-expression of these proteins through the hormonally responsive MMTV promoter. At the present time, MMTV-CrkII transgenic mice have been characterized and a manuscript is in preparation for submission to *Oncogene* in April (Appendix 8). The Crk protein can be detected through immunohistochemistry, RT-PCR (using primers specific for the transgene) and Western blot analysis. Of the 7 founder lines, 2 were kept for further analysis.

In each line studied, abnormal mammary development was found. For instance, five to twelve week old virgin mice were found to have delayed ductal outgrowth, compared to their negative FVB littermates. In these mice at 12 weeks of age, terminal end buds are persistent and disorganized, and have not yet reached the edges of the fat pad, whereas in their wild-type litter mates, these buds are regressed and the fat pads are filled with branching ducts. This phenotype is similar to other mouse models, such as MMTV-EphB4, MMTV-Cripto and MMTV-Krct (17, 18, 19). Although many other mouse models which show this phenotype do not examine how delayed outgrowth occurs, I have shown that it appears to be the result of increased collagen surrounding the terminal end buds. This was determined by Trichrome staining and indicates a slowed development of the mammary gland. However, whether CrkII induces the formation of

collagen or inhibits its breakdown remains to be determined. No significant changes were found in terms of cellular proliferation, apoptosis or differentiation in the terminal end buds.

In other mice of these lines (at 20 weeks and 1 year of age), there is increased ductal branching, suggesting enhanced proliferation of the epithelial branches and this is accompanied by increased PCNA staining. MMTV-p130Cas mice show a similar branching phenotype (20). Western blot analysis did not reveal any significant changes in the Akt, Erk or JNK pathways, however it is possible that any changes in the activation of these pathways may be missed at this particular time point. Lobular hyperplasia was found in aging virgin mice but pregnancy, lactation and involution appear normal in these mice.

Tumor development was monitored in both virgin and multiparous females, with a combined tumor incidence of 35% thus far and a tumor latency of approximately 14 months. These initial results are promising, as many other adaptor and scaffold proteins over-expressed in the mammary gland were unable to induce tumorigenesis (ie. MMTV-Grb2, MMTV-p130Cas, MMTV-Gab2) (20, 21, 22). The CrkII tumors have been transplanted into FVB mice and cell lines have been established. One CrkII tumor has been identified as a fibroadenoma. The tumor is positive for CrkII, CK8, CK14, PCNA, vimentin and smooth muscle actin via immunohistochemistry. Another CrkII tumor has been identified as squamous adenocarcinoma, with a high mitotic index and the presence of cytokeratin pearls. The pathology of the CrkII tumor is similar to MMTV models in which the Wnt pathway is over-expressed, suggesting that it may be a basal phenotype, which is often associated with poor prognosis in human cases (23, 24). Staining was positive for CK14+ and smooth muscle actin (myoepithelial markers), CK8 (epithelial marker) as well as CK6+ (a putative progenitor marker) suggesting that this tumor is indeed basal-like and is of a mixed lineage. Finally, expression profiling specific for the transgene confirms that the tumor is indeed positive for the Crk transgene. These findings are important, as basal tumors in human breast cancer patients are often associated with poor prognosis (24). I will be flying to Sacramento in March to meet with the world renowned pathologist Dr. Robert Cardiff, to go over the hyperplastic and tumor phenotypes found in these CrkII mice to further evaluate the data.

As previously mentioned, a division of our lab, the Breast Cancer Functional Genomics Group (BCFGG), have generated gene expression profiles from laser capture microdissected tumor and matched-normal epithelium from human breast cancer patients. Greg Finak, another PhD student in our lab, has identified that CrkL (which is 60% homologous to CrkII) is highly expressed in basal tumors compared to other tumor types. However, CrkII is highly expressed in several different subsets of human breast cancer. A collaboration with Dr. Anne Marie MesMasson's lab has been set up in order to do immunohistochemical staining for CrkII on human tissue microarrays to examine whether CrkII is over-expressed in basal versus luminal breast cancers and whether over-expression of CrkII is linked to decreased survival.

Since our mouse model leads to hyperplasia and tumor development at a long latency and low penetrance, it suggests that other additional genetic events are required to drive tumor development. We will be crossing our mice with the knock-in ErbB2 mouse model as well as the p53 null mice to determine whether CrkII can synergize with these models to drive tumor progression and metastasis.

The MMTV-CrkI mice have been generated and 13 founder lines were established. Of these lines, 4 were terminated as the mice were not breeding properly. Expression profiling was carried out to determine lines which show high levels of CrkI expression. In contrast to the CrkII mice, 12/16 of CrkI animals at 12 weeks of age have normal ductal outgrowth, whereas only 4/16 showed delayed ductal outgrowth which is similar to CrkII mice. A potential lactational defect in these mice is currently being examined as there are patches of the mammary gland which do not appear to be producing milk. Lactation in CrkII mice were normal, suggesting a key difference between these two Crk isoforms. Although their expression profiles should be compared to the levels of expression of CrkII mice, this suggests that both CrkI and CrkII proteins have variable roles in mammary gland development. Interestingly, CrkI staining via immunohistochemistry is localized at cell-cell junctions, unlike CrkII which is mainly nuclear.

The MMTV-CrkI mice had trouble breeding so we kept 3 lines to set up for multiparous and aging virgin cohorts. Thus far, two founder mice have developed tumors, one of which metastasized to the lungs. The pathology and tumor characteristics are currently being examined in collaboration with Dr. Robert Cardiff. Furthermore the role of CrkI in all aspects of mammary gland development (puberty, pregnancy,

lactation and involution) are also being examined. Thus, in the upcoming year, I hope to set up all the animals I need to examine the role of CrkI in mammary gland development as well as tumor development.

Lastly, the MMTV-CrkL construct has been made. The tagged protein has been shown to interact with the endogenous CrkL binding partner, Gab1, which is enhanced upon HGF stimulation. The over-expression of CrkL and Gab1 leads to Rac activation, confirming previous reports. The construct for this mouse has been made and the expression profiling is currently being carried out.

## **KEY RESEARCH ACCOMPLISHMENTS**

- \* Crk proteins are important regulators of breast cancer migration and invasion
- \* High CrkI/II levels affect mammary gland development
- \* Over-expression of CrkII can lead to breast cancer development
- \* Tumors derived from CrkII may be derived from epithelial progenitor cells
- \* Over-expression of Crk proteins in human breast cancer may be associated with poor prognosis disease

## **REPORTABLE OUTCOMES**

### **Manuscripts**

Fathers, KE, Monast, A, Rodrigues, SP, Cardiff, R, Park, M. (2008). Over-expression of CrkII leads to abnormal mammary gland development and breast cancer. Goal is to be submitted to *Oncogene* April 2008.

Rodrigues, SP<sup>^</sup>, Fathers, KE<sup>^</sup>, Chan, G, Zuo, D, Halwani, F, Meterissian, S, Park, M. 2005. CrkI/II function as key signalling nodes for migration and invasion of cancer cells. *Molecular Cancer Research*. 3 (4):183-94  
<sup>^</sup> - Both authors contributed equally to this work.

### **Abstracts**

Fathers KE, Monast A, Rodrigues S, Cardiff R, Park, M. Over-expression of CrkII leads to abnormal mammary gland development, lobular hyperplasia and breast cancer. Gordon Research Conference - Mammary Gland Biology. June 1-6, 2008. Il Ciocco, Barga Italy.

Fathers KE, Monast A, Rodrigues S, Park M. Over-expression of CrkII leads to abnormal mammary gland development and a basal cancer phenotype. Mechanisms and Models of Cancer. August 8-12<sup>th</sup>, 2007. La Jolla, California, USA.

Fathers KE, Monast A, Rodrigues S, Park M. MMTV-CrkII mice display a defective ductal outgrowth phenotype in peripubertal mice. Gordon Research Conference - Mammary Gland Biology. May 28-June 2, 2006. Il Ciocco, Barga Italy.

Rodrigues, SP, Fathers, KE, Chan, G, Park, M. 2004. Crk adaptor proteins play an essential role in cellular migration and invasion in multiple human cancer cell lines. Twentieth Annual Meeting on Oncogenes. Frederick, USA

Fathers, KE, Rodrigues, SP, Park, M. Crk adaptor proteins play a key role in cellular migration and invasion in human cancer. 2005. American Society of Cellular Biology - Systems Integration in Directed Cell Motility. Seattle, USA

Fathers, KE, Rodrigues, S, Park, M. 2006. Crk adaptor proteins play a key role in cellular migration and



invasion in human cancer. Canadian Breast Cancer Research Alliance - Reasons for Hope. Fourth Scientific Conference. Montreal, Canada

Fathers, KE, Monast, A, Rodrigues, SP, Park, M. 2006. MMTV-CrkII mice display a defective ductal outgrowth phenotype in peripubertal mice. Gordon Research Conference - Mammary Gland Biology. Barga Italy.

## **Presentations**

Fathers, KE. Crk adaptor proteins act as key signaling integrators in human breast cancer. Molecular Oncology Group Seminar Series. McGill University, Montreal, Canada.

## **Animal Models**

MMTV-CrkLV5

MMTV-CrkII

MMTV-CrkI

## **Cell Lines**

MDA-231 breast cancer cell lines stably expressing shRNA targeting CrkI/II

MDA-231 breast cancer cell lines stably expressing shRNA targeting CrkL

MDA-231 breast cancer cell lines stably expressing shRNA targeting CrkI/II and CrkL

MMTV-CrkII tumor cell lines, derived from MMTV-CrkII tumors

MMTV-CrkII/ErbB2 tumor cell lines, derived from ErbB2/CrkII + tumors

## **CONCLUSION**

The present study demonstrates that Crk adaptor proteins play an important role in integrating signals for migration and invasion of highly malignant breast cancer cell lines. This has important implications as elevated levels of Crk are observed in various human cancers, such as breast. Hence, our findings demonstrating a role for Crk as a key integrator of signals for cell migration and invasion identifies a potential role for Crk in metastatic progression. Future work from this project will show the importance of Crk adaptor proteins during metastasis, which is the rate-limiting factor in cancer treatment. Furthermore, this project suggests that over-expression of Crk leads to hyperplastic lesions as well as tumor development. Thus, our study on Crk adaptor proteins has the potential to identify some of the molecular events, which can occur during breast cancer metastasis and tumorigenesis. This may provide information, which could be used to develop effective anti-metastatic treatments for breast cancer.

## **“SO WHAT”**

Metastasis is a major cause of morbidity and mortality in human malignancies, and is the driving force behind the incessant pursuit of “anti-metastatic” and adjuvant therapies. For instance, patients with metastatic breast cancer have a median survival of only 2 to 3 years and twenty percent of the patients who present with bone metastases have only a five year survival from the time of diagnosis (25). As a result, current therapies for metastatic breast cancer are aimed at improving palliative care rather than complete remission (25). Further progress in this field of research may be achieved through a better understanding of the various molecular processes defining the complexity and multi-step nature of tumor cell dissemination, otherwise known as the metastatic cascade. My research has shown that Crk adaptor proteins are key integrators for cellular migration and invasion in various breast cancer cell lines, regardless of their inherent mutations and upstream signals. This suggests that Crk adaptor proteins may be viable targets for the metastasis, as cellular migration and invasion are key components of the metastatic cascade. Through my future work using in vivo metastatic mouse models as well as microarray analysis, I may be able to dissect

signaling pathways responsible for metastasis. This will be an important step towards developing targeted therapeutics for breast cancer metastases.

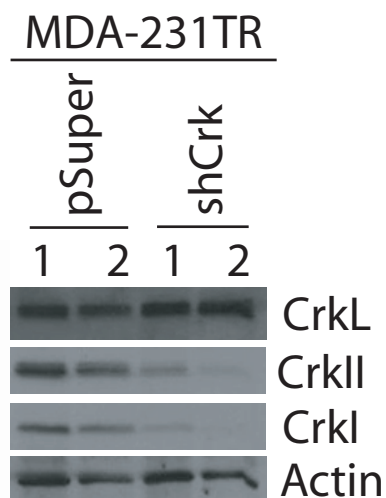
In addition to identifying a novel role for Crk adaptor proteins in breast cancer metastasis, I have shown that CrkII is linked to the formation of hyperplasias and basal-like breast cancers. The low penetrance and low latency suggests that over-expression of CrkII may not drive tumor development, but perhaps expression of Crk is required for tumor progression. Combining our work with the previous data from our lab, which showed that Crk can convert a non-invasive response to an invasive response, suggests that Crk may not necessarily need to be elevated to be important for tumor development. In reality, Crk may synergize with several upstream signals to drive tumor progression rather than driving tumor progression itself. For instance, I have shown that loss of Crk inhibits migration and invasion of several basal breast cancer cell lines, signifying that Crk may be a key signaling node for multiple tumors, regardless of their inherent mutations. As these “basal” breast tumors are triple negative (Her2, ER, PR negative), treatment options for these tumors are limited. Thus, the need for identifying potential targets for these tumors is important. Although preliminary array analysis shows that Crk is over-expressed in multiple breast cancer subtypes, this will be confirmed through tissue arrays. Furthermore, the development of MMTV-CrkI and MMTV-CrkL mouse models as well as the shRNA-mediated responses using in vivo metastatic mouse assays will further illustrate whether these proteins are important for a basal tumor phenotype. Thus, the data generated from this study has the potential to identify Crk adaptor proteins as valuable targets for these types of breast tumors.

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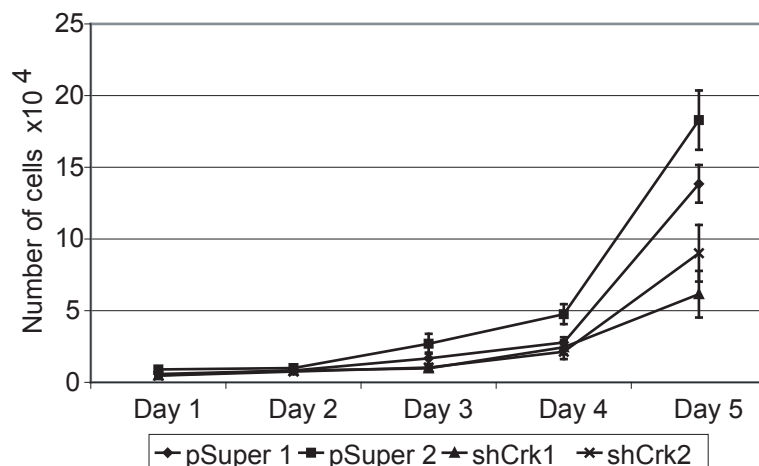
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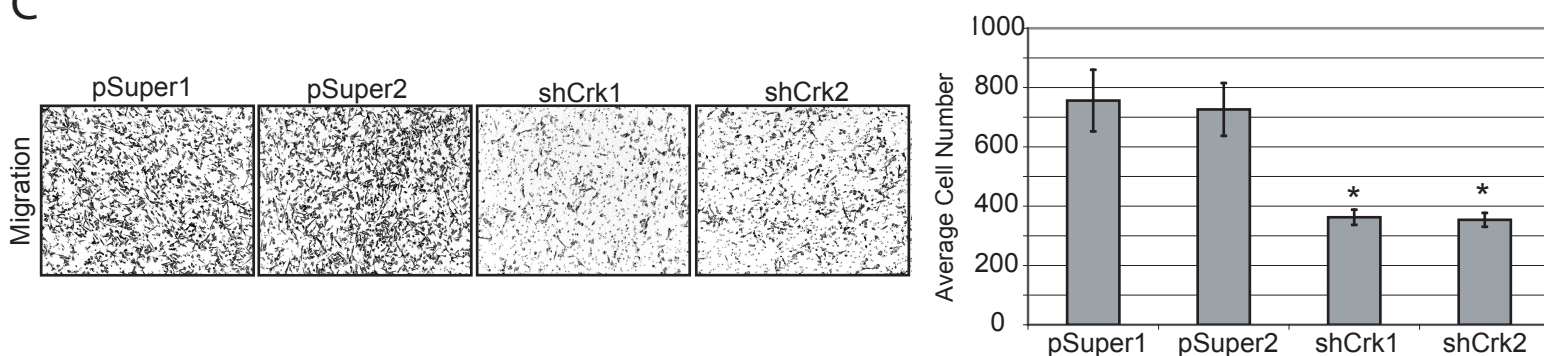
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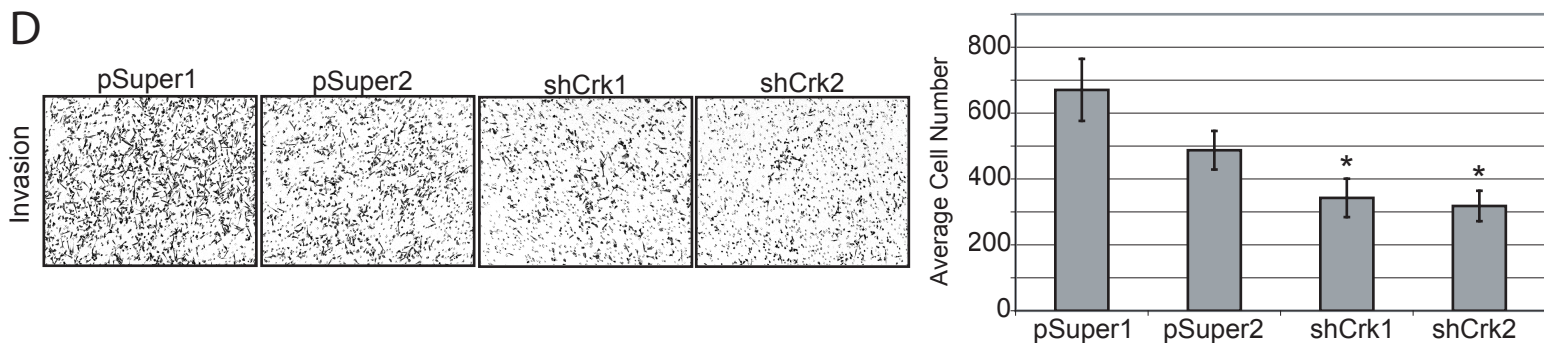
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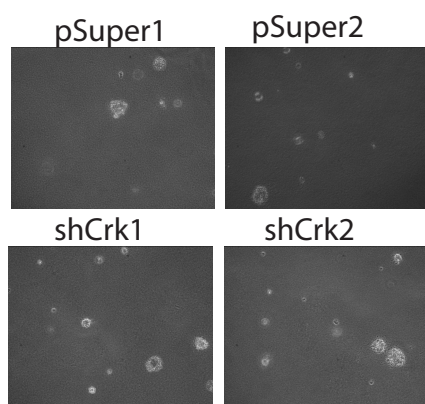
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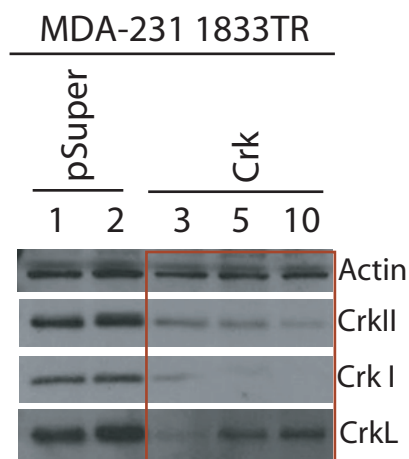


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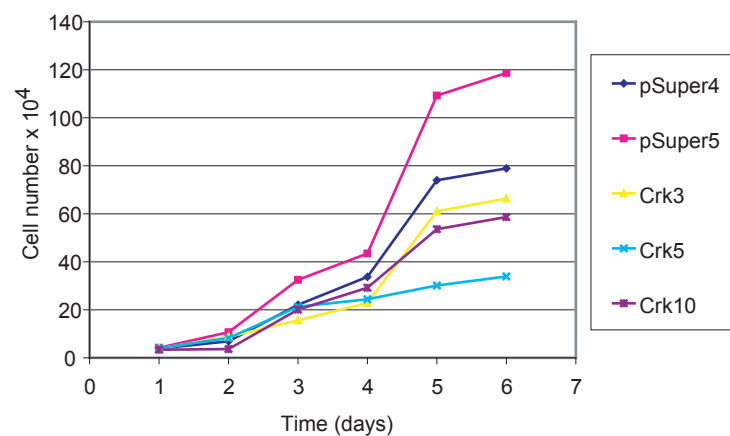


Appendix 1: Summary of CrkI/II stable clones isolated from MDA231TR cells (A). Cell doubling assay (n=3) (B). Migration and invasion of Crk clones relative to controls, n=3, p<0.05 (C, D). Results from soft agar assay (E).

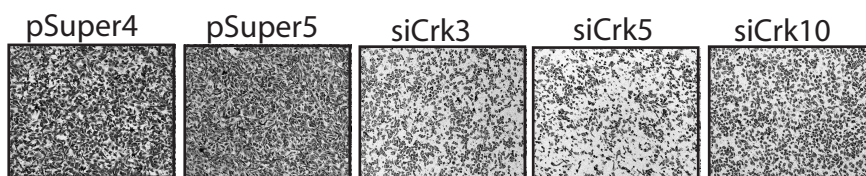
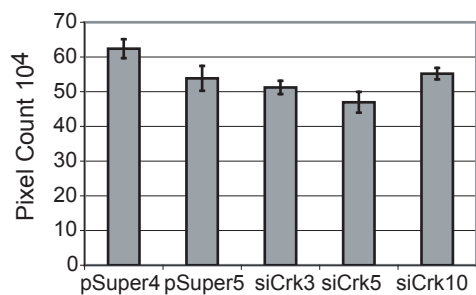
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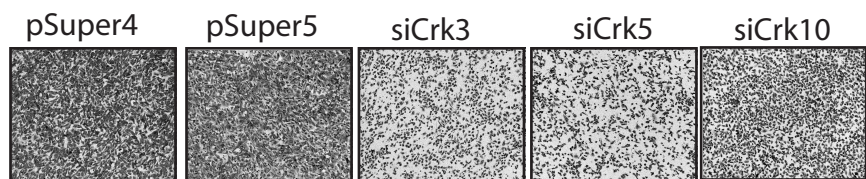
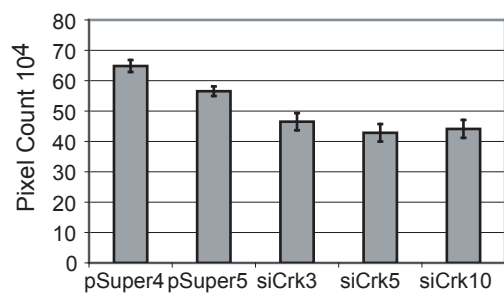
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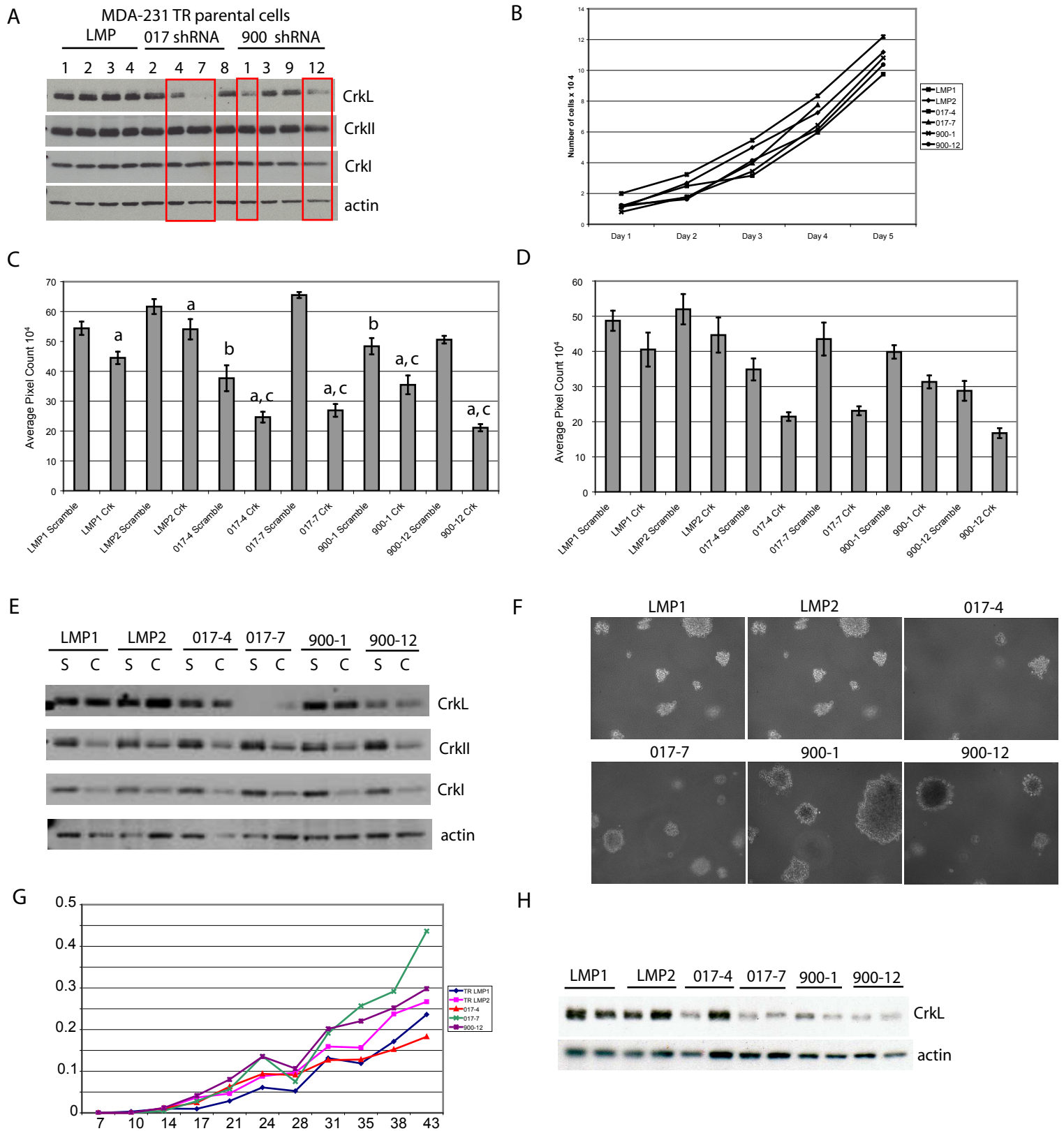
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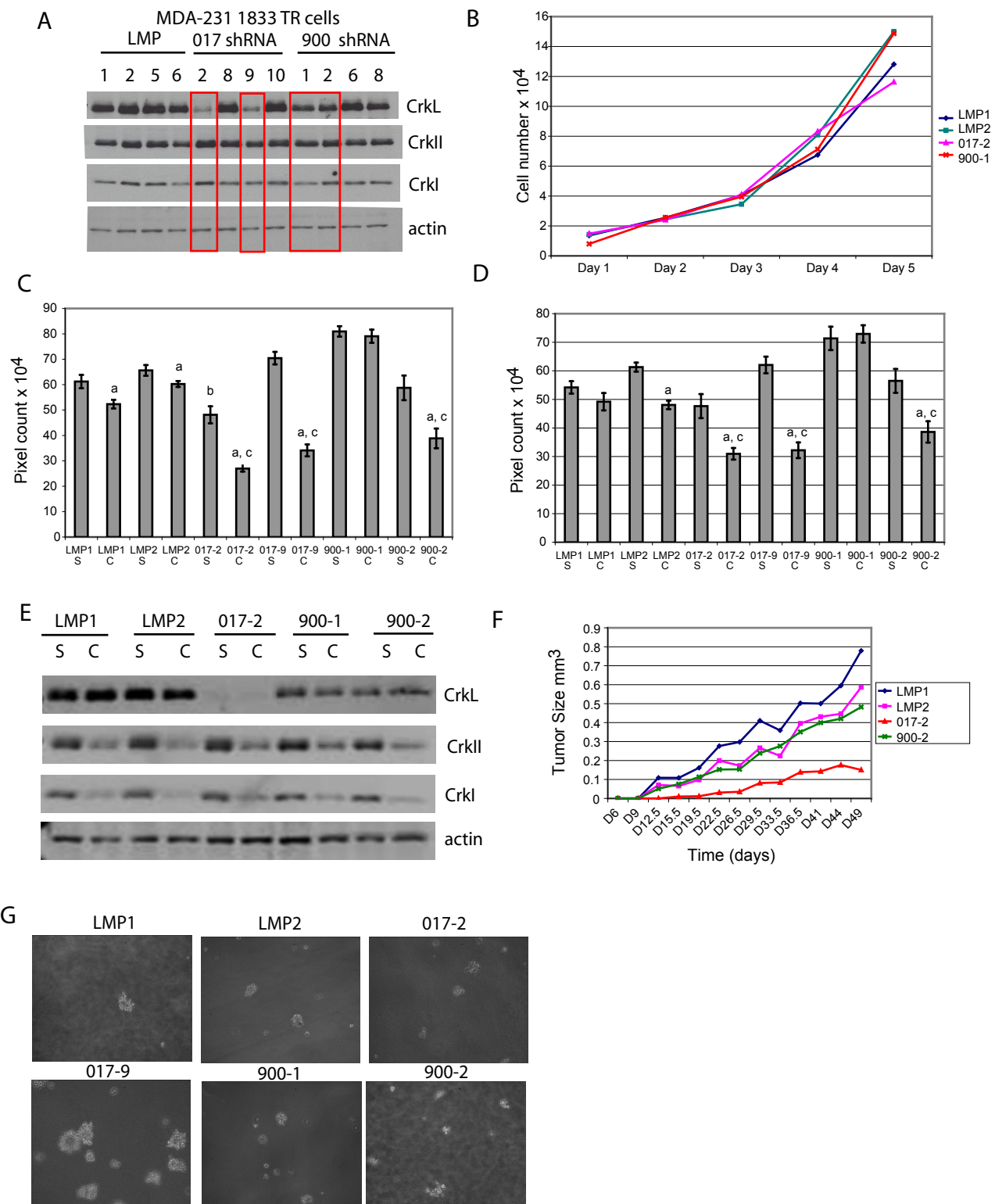
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Appendix 2: Summary of Crk stable clones isolated from MDA231TR cells (A). Cell doubling assay (n=3) (B). Migration and invasion of Crk clones relative to LMP controls, n=3 (C, D). New analysis methods are currently being tried to more accurately quantitate the results. Visual images suggest that loss of Crk does inhibit cellular migration and invasion. Soft agar assay not performed yet.



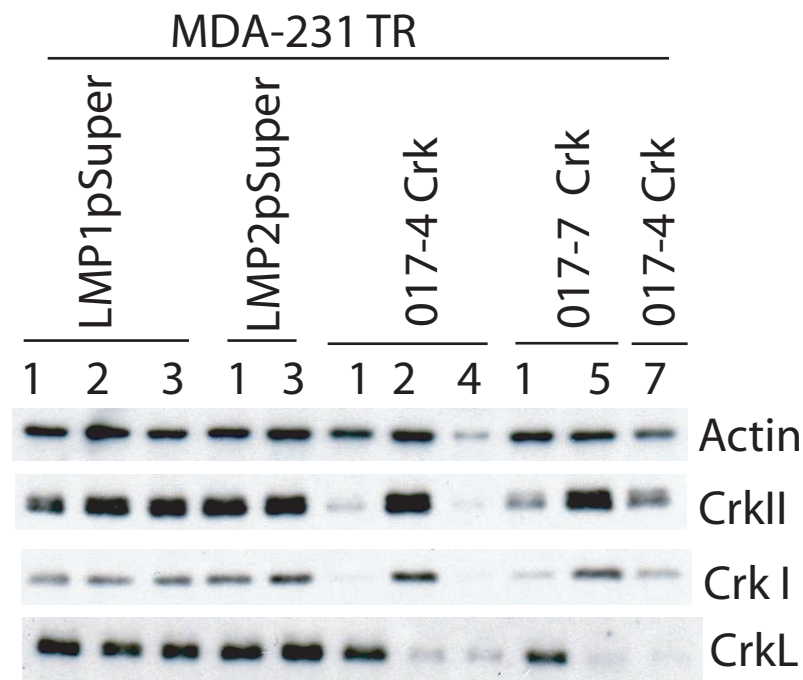
Appendix 3 : Summary of CrkL stable clones isolated from MDA231TR cells (clones further characterized in red) (A). Cell doubling assay for CrkL clones, n=2 (B). Synergistic effects of stable CrkL clones with transient CrkI/II siRNA on migration and invasion, n=3 (C, D). Western blot confirming CrkI/II and CrkL triple knockdown (E). Soft agar assay results for stable clones, n=2 (F). Tumour growth curves for mammary fat pad injections with CrkL stable clones (G) with confirmation of knockdown in tumors (H).



Appendix 4: Summary of CrkL stable clones isolated from MDA2311833 TR cells (clones further characterized in red) (A). Cell doubling assay for CrkL clones, n=2 (B). Synergistic effects of stable CrkL clones with transient CrkI/II siRNA on migration and invasion, n=3 (C, D). Western blot confirming CrkI/II and CrkL triple knockdown (E). Tumor growth curves from mammary fat pad injections with CrkL stable clones versus controls (F). Soft agar assay results for stable clones, n=2 (G).



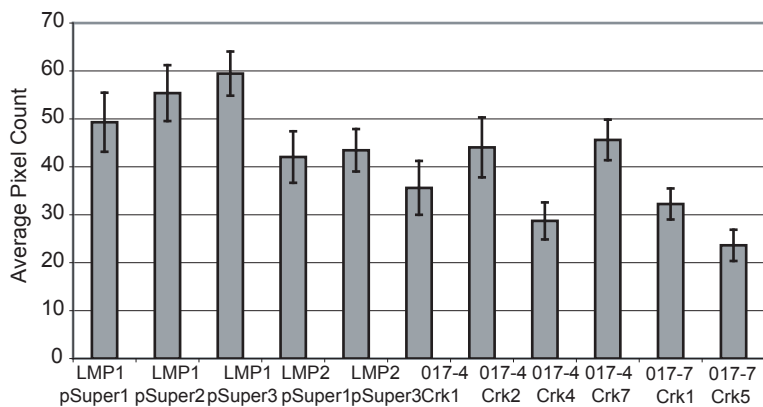
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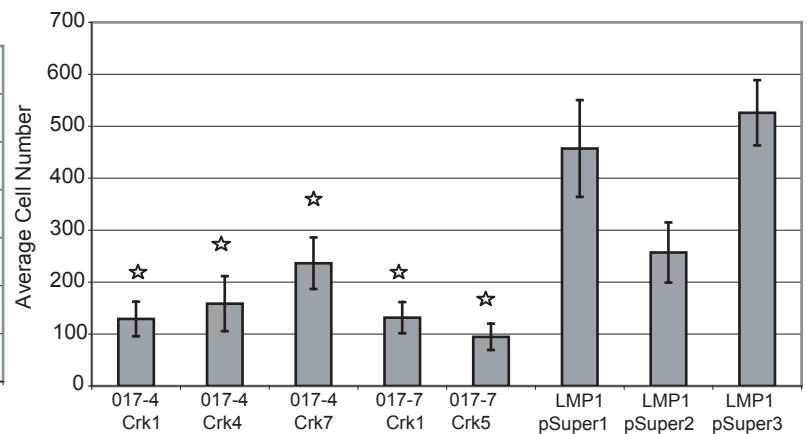
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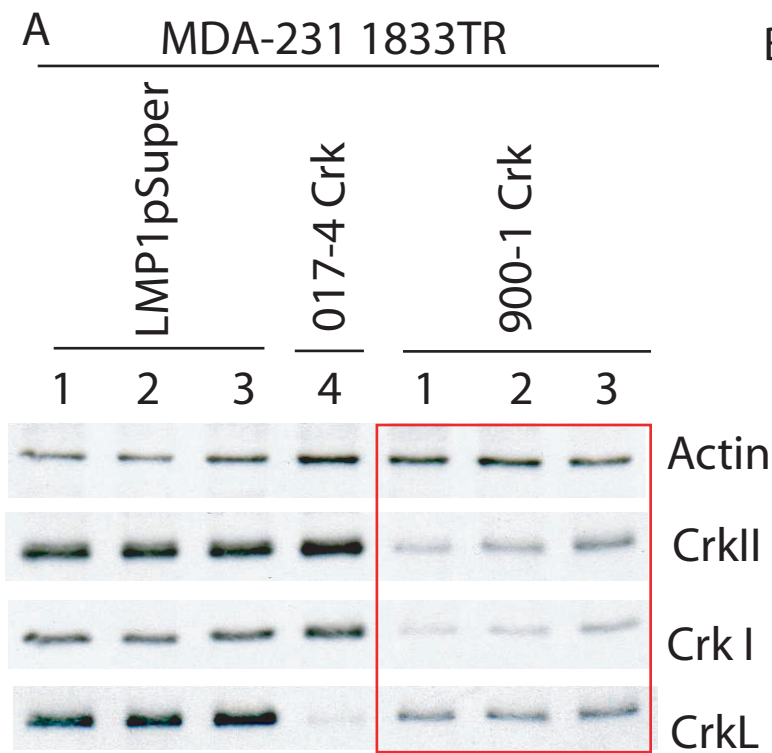


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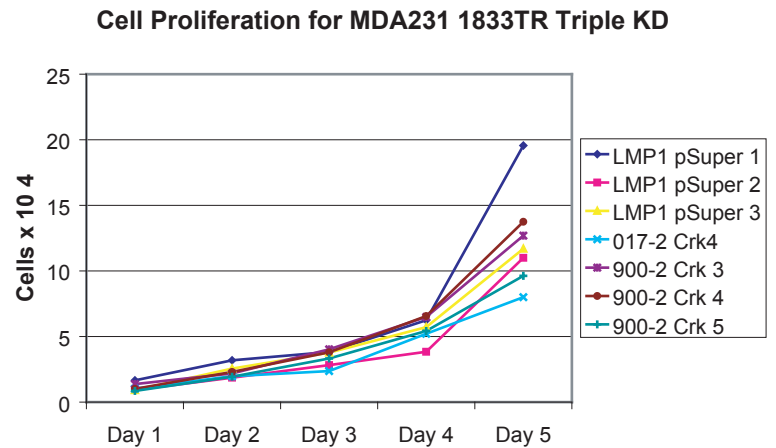


Appendix 5: Summary of Crk stable clones isolated from MDA231 TR cells which lost their triple knockdown status in culture after a long period of time (A). New clones currently being selected. Cell proliferation assay to be done with new clones (B). Migration and invasion of Crk clones relative to LMP controls, n=3 using the clones obtained in A(C, D). The migration data was using an older method of analysis (pixel count) (C) whereas the invasion was using a cell counting approach, which shows significant differences (\*) when compared to LMP1pSuper1 (t test,  $p < 0.05$ ) (D). These two methods are currently under review for the best form of analysis.

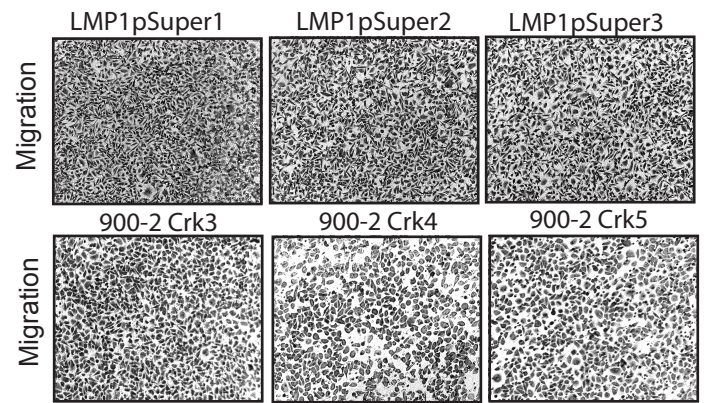
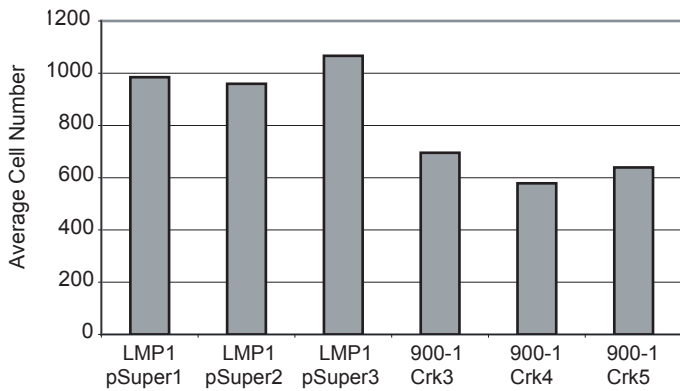




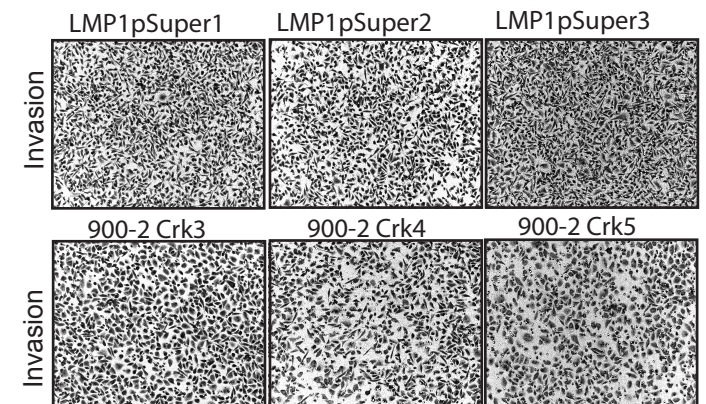
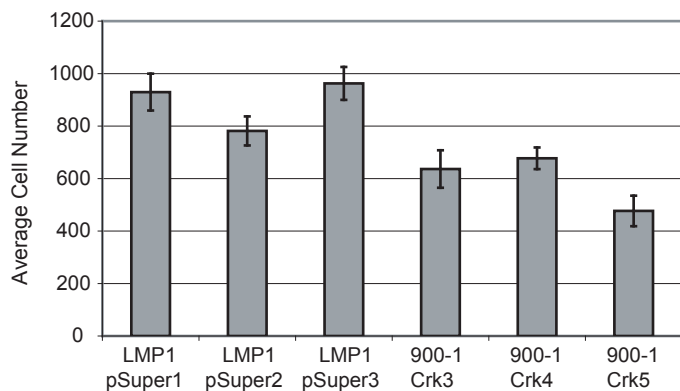
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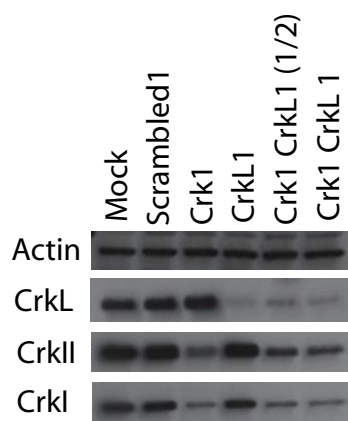


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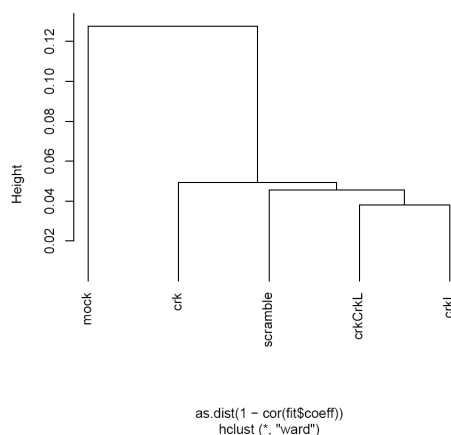


Appendix 6: Summary of CrkI/II/L stable clones isolated from MDA2311833 TR cells (clones further characterized in red) (A). Preliminary cell proliferation assay (B). Migration and invasion of Crk clones relative to LMP controls, n=2 for migration whereas n=3 for invasion (C, D).

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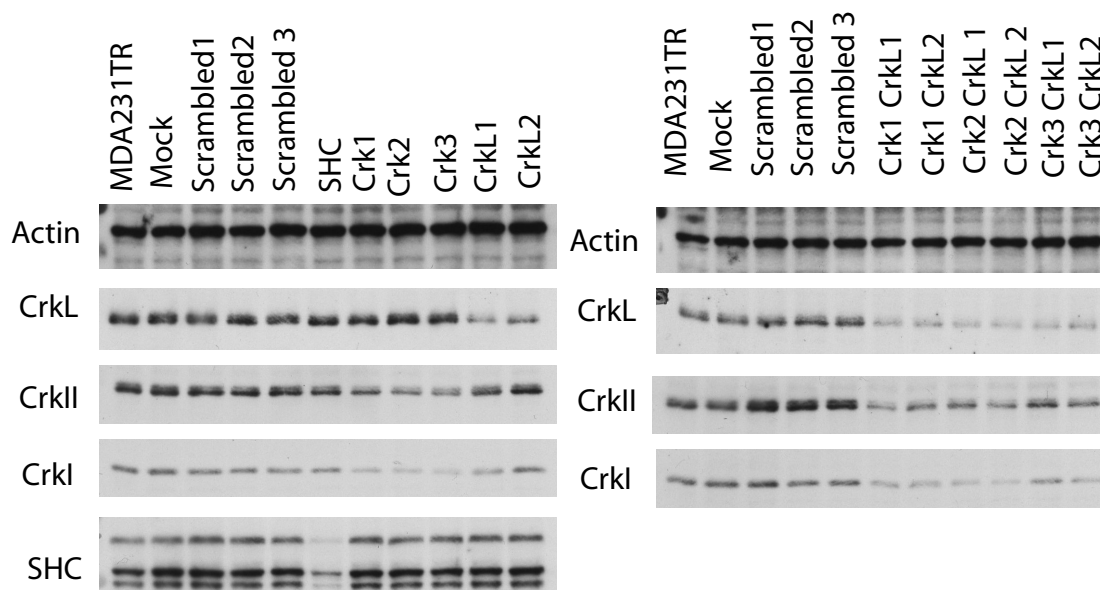
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Condition	Gene Name	Fold Increase	Gene Function
CrkL vs scramble	CXCL11	2.8x	Thought to be angiostatic
	CDC42SE2	-2.5x	Mediates Cdc42 signaling
	Calponin 2	2.1x	
	CrkL	-4x	As expected!!!!
Crk/II vs scramble	CSF3	-3.6x	
	KIF2A	-1.6x	Involved in fast axonal transport of organelles
Crk/CrkL vs scramble	ADAMTS14	3.1x	Targets and processes the ECM
	Vasorin	4.1x	Attenuates TGF-beta signaling
	ARHGD1B	-1.9x	Shuttles Rho GTPases between membrane and cytosol
	PAK1IP1	-2.1x	Negative regulator of PAK

D



Appendix 7 - Preliminary microarray results. The Western blot confirming knockdown of a preliminary experiment using a small subset of conditions (Mock, Scramble siRNA, Crk siRNA, CrkL siRNA and Crk/CrkL siRNA (used at 2 different concentrations) (A). Non-biased clustering analysis of the array results from the preliminary experiment (B). Small list of potentially relevant genes found in the preliminary microarray experiment (compared to Scramble but also altered relative to Mock (C). Western analysis showing successful knockdown of all conditions for one microarray experiment to be performed for the official analysis (D).

## **Over-expression of CrkII leads to abnormal mammary gland development, lobular hyperplasia and breast cancer**

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**Running title:** CrkII in mammary development and tumorigenesis

**Key words:** adaptor protein, breast cancer, terminal end buds, transgenic mice

### **Abstract**

The Crk protein was originally isolated as the oncogene fusion product of the CT10 chicken retrovirus. The cellular homologues of v-Crk, include CrkI, CrkII and CrkL. CrkI/II proteins are elevated in breast cancer, however, the question of whether Crk adaptor proteins can lead to breast cancer has not been addressed. We created a transgenic mouse model that allows the expression of CrkII through the MMTV promoter. Transgenic mice undergoing puberty were found to have delayed ductal outgrowth, compared to their wildtype littermates. This indicates a slowed development of the mammary gland and is characterized by increased collagen surrounding the

terminal end bud. The fat pad is eventually filled and in older mice, there is precocious ductal branching associated with increased proliferation. Focal mammary tumors appeared in x% of the animals examined with a latency of approximately 14 months. MMTV-CrkII tumors express high levels of Crk as well as cytokeratin markers 6, 8 and 14, suggesting that they may be of mixed lineage. This study provides the first demonstration of a potential role for the CrkII adaptor protein in integrating signals for mammary gland development and breast cancer progression in vivo, which has important implications for elevated CrkII in human cancer.

## **Introduction**

Crk was originally isolated as the oncogene fusion product of the CT10 chicken retrovirus (v-Crk) in chicken fibrosarcomas (1). Cellular homologues of v-Crk, include c-Crk, which encodes two proteins (c-CrkI and c-CrkII) through alternative splicing, and c-CrkL. Crk proteins are composed of Src homology 2 (SH2) and Src homology 3 (SH3) domains (2). CrkI contains one SH2 domain and one SH3 domain, whereas CrkII also contains a linker region (containing a Y221 negative regulatory site) and a second SH3 domain at the C-terminus (2). CrkL is similar to CrkII with one SH2 domain and 2 SH3 domains, and shares 60% homology with CrkII (3). Although CrkI, CrkII and CrkL are ubiquitously expressed in several tissue types, CrkL is highest in hematopoietic tissues, and CrkI is expressed at lower levels than CrkII (3, 4). The Crk adaptor proteins have no enzymatic activity but rather play an important role during cellular signalling by mediating the formation of protein-protein complexes (2). In response to extra-cellular stimuli, Crk has been implicated in the activation of several effector proteins involved in cellular migration, invasion, adhesion, proliferation, apoptosis or phagocytosis,

depending on the signal transduction pathway activated (2). For instance, in response to HGF, Crk adaptor proteins are required for dispersal of organized epithelial colonies, the formation of lamellipodia as well as the breakdown of adheren junctions, all of which are events critical for tumor cell dispersal, invasion and hence, cancer progression (5). The over-expression of Crk promotes an invasive phenotype regardless of upstream signalling, identifying Crk as a potential key regulator of upstream signals for cell invasion (6). Moreover, Crk proteins are elevated in multiple types of cancer, including lung carcinoma, glioblastoma, leukemia and breast cancer (7-10).

All three Crk proteins (CrkI, CrkII, CrkL) have been shown to be involved in transformation, albeit to varying degrees. CrkI, which most closely resembles v-Crk, has been found to have the highest transforming potential. Fibroblast cells expressing CrkI proliferate in soft agar and form tumors in nude mice (11). Although CrkII was thought to have no transforming activity (11), a more recent study has shown that over-expression of CrkII in NIH3T3 fibroblasts and various mouse embryo fibroblasts can induce growth in soft agar, suggesting that CrkII can play a role in anchorage independent growth, whichh is one component of cell transformation (12). Over-expression of CrkL also leads to anchorage independent growth in fibroblasts; furthermore, a mouse model over-expressing CrkL through its endogenous promoter was associated with an increased incidence of tumor formation, including both hematopoietic and epithelial cancer types (13, 14). Thus, there is now evidence that all three Crk proteins are capable of transforming cells, implicating these adaptor proteins in cancer.

Although CrkII has been shown to be elevated in various types of cancer, including breast cancer, and can lead to anchorage independent growth, the ability of

CrkII to induce mammary tumorigenesis in vivo has yet to be elucidated. Much of the in vitro studies on CrkII have focused on fibroblasts cells, however, CrkII is well expressed in the epithelium of many tissues (4). Furthermore, in addition to studying the effects of CrkII over-expression in the mammary epithelium, its effects on mammary gland development and remodeling are also largely unknown. Thus, in order to examine the importance of CrkII in both tumor progression and mammary gland development, we derived transgenic mice expressing CrkII in the mammary epithelium, under the transcriptional control of the mouse mammary tumor virus promoter (MMTV).

## **Results**

### **Over-expression of CrkII in the mammary epithelium leads to defects in ductal outgrowth**

To determine the effects of over-expression of CrkII on mammary gland physiology and tumorigenesis, MMTV-CrkII transgenic mice were generated (Figure 1A). Five positive founder lines were further characterized for transgene expression. Mammary glands from both virgin and lactating female mice were examined for expression levels of the transgene by Western blot and RT-PCR analysis (Figure 1B, C). The MMTV promoter induces high expression during pregnancy and lactation, although the transgene is expressed in the virgin mammary gland as well (19, 20). Expression of the CrkII transgene using primers specific for the transgene SV40 polyA signal indicates that the transgene is expressed well in each transgenic line in both virgin and lactating mice (Figure 1C). Moreover, although the antibody recognizes endogenous protein, it is clearly evident that CrkII is higher in transgenic mice than wildtype FVB mice in lactating animals (Figure 1B). MMTV-CrkII lines (6396 and 6398) with the highest levels of expression specific for the transgene at the mRNA level (Figure 1C) were used

for subsequent studies on tumorigenesis and mammary gland development. Immunohistochemistry on sections from paraffin embedded mammary epithelium reveals strong but heterogeneous Crk expression in both the nucleus and the cytoplasm of luminal epithelium in the transgenic mice when compared to control FVB mice (Figure 1D).

To determine whether over-expression of CrkII in the mammary epithelium could perturb normal mammary gland development, wholemounts of virgin mammary glands were examined. In all 5 founder lines studied, abnormal mammary development was found (Figure 2B). For instance, five to twelve week old virgin mice were found to have delayed ductal outgrowth, compared to their negative FVB littermates (Figure 2A). During puberty, the mammary epithelium normally infiltrates the mammary fat pad by proliferation and differentiation of the terminal end buds (15). By the end of puberty at 10 weeks of age, the entire fat pad is filled with branching epithelium followed by the regression of the terminal end buds (15). Ductal outgrowth is delayed in CrkII transgenic mice. The suppression of ductal outgrowth was most significant at 7-10 weeks of age however, in MMTV-CrkII mice at 12 weeks of age, terminal end buds were still persistent and disorganized, and had not yet reached the edges of the fat pad, whereas in their wild-type litter mates, these buds were regressed and the fat pads filled with branching ducts (Figure 2A). The effect of CrkII on mammary ductal outgrowth was quantified by determining the percentage of the fat pad filled. Transgenic mice with over-expression of CrkII at 12 weeks of age displayed an inhibition of ductal outgrowth from ~4-25% depending on the line examined (Figure 2B). Inhibition of ductal outgrowth was most pronounced at 10 weeks of age, with an inhibition of 31% (Figure 2B). Histological

sections of MMTV-CrkII mammary glands revealed disorganized and thickened stroma surrounding the terminal end buds compared to normal FVB littermates (Figure 2C). This was also characterized by increased collagen surrounding the terminal end buds as determined by Trichrome staining and indicates a delayed development of the mammary gland (Figure 2C). Staining for Crk protein is found in the terminal end bud structures of transgenic mice but is relatively absent in FVB littermates (Figure 2C). The suppression of ductal outgrowth did not coincide with significant changes in cell proliferation, apoptosis, or differentiation (through staining of PCNA, TUNEL, CK8, and CK14), which are all active processes within the terminal end bud structures (16) (Supplemental data). Therefore, multiple lines of transgenic mice with elevated levels of CrkII in the mammary epithelium display a delay in development during puberty by suppressing the outgrowth of epithelial ducts through an increase in stroma surrounding the ducts.

**CrkII over-expression leads to enhanced mammary gland branching and hyperplasia characterized by increased proliferation.**

Once the glands have fully developed, there is a discreet Crk dependent phenotype, characterized by increased ductal branching (Figure 3A). This is accompanied by increased PCNA staining, supporting enhanced proliferation of the epithelial branches as well as elevated Crk staining in the branching epithelium (Figure 3A). To determine whether the observed mammary epithelial abnormalities reflected the activation of known targets of Crk, we assessed Crk binding proteins in protein lysates by Western blot analysis from mammary glands of 20 week old mice. No significant changes were found amongst all seven of the MMTV-CrkII mice examined in the Akt, Erk or JNK pathways



(data not shown). However, it is possible that alterations in the activation of these pathways may be missed at this time point.

**CrkII over-expression leads to hyperplasia in adult virgin mice but does not disrupt other stages of mammary gland development.**

In aging virgin transgenic mice at approximately one year of age, lobular hyperplasia was found (Figure 3B). Other stages of mammary gland development have also been examined (Supplemental Figure 2). No significant changes in lactation at Day 15 were found. Secondly, mice examined at Day 10 and Day 18 of pregnancy showed no alterations when assessed by wholemount analysis and histology. Finally, no significant perturbations were noted in mice undergoing involution at Day 3 and Day 20 (Supplemental data). Hence, CrkII over-expression in the mammary epithelium disrupts early development, promotes enhanced branching and hyperplasia but does not lead to obvious changes during lactation, pregnancy and involution.

**Elevated levels of CrkII predisposes the mammary epithelium to the development of basal-like breast cancer.**

To determine whether over-expression of CrkII could lead to the development of mammary tumors, two cohorts of mice (virgin and multiparous) were established for the 6396 and 6398 transgenic lines. In the two lines studied, tumor development was monitored in both virgin and multiparous females, with a combined tumor incidence of x% and a tumor latency of approximately x days (15 months) (Table 1). By contrast, the tumor incidence in female FVB control mice was x%. The induction of mammary tumors in the MMTV-CrkII mice was accompanied by an increase in the overall levels of Crk protein (Figure 5). The tumors from the MMTV-CrkII mice were of a diverse phenotype,

including fibroadenomas and squamous adenocarcinoma (Figure 5). No metastatic lesions were found in any animals. The fibroadenomas consisted of several differentiated epithelial cells (CK8, CK14) interspersed within regions of mesenchymal-like cell populations (vimentin, smooth muscle actin) (Figure 5). The squamous adenocarcinoma, characterized by a high mitotic index and the presence of cytokeratin pearls, stained positive for CK14+ and smooth muscle actin (myoepithelial markers) as well as CK6+ (a putative progenitor marker) and CK8+ (luminal marker) suggesting that this tumor is of a mixed lineage, and thus considered basal-like (Figure 5). Quantitative real-time PCR specific for the transgene confirms that the tumor is indeed positive for the Crk transgene (data not shown).

Thus, over-expression of CrkII can lead to tumor development, albeit at a low incidence, suggesting that other genetic events are required for tumor onset. The tumors which do form, express various cytokeratin markers, suggesting that they may arise from a progenitor cell.

## **Discussion**

This study provides direct evidence that elevated levels of CrkII in the mammary epithelium of transgenic mice can interfere with normal mammary gland development and induce mammary epithelial tumors, albeit with a low incidence. The CrkII transgene exhibited expression that is characteristic of the MMTV promoter, with higher levels of expression in the lactating gland compared to the virgin state (19, 20). The CrkII transgene expression was detected at both the mRNA and protein levels. Immunohistochemistry revealed heterogeneous staining for the transgene throughout the mammary epithelium and even within ducts. This most likely reflects the nature of the

MMTV promoter, as several other MMTV mouse models also show heterogeneous staining (18, 19). CrkII was localized in both the nucleus and cytoplasm of the mammary epithelial cells. Although most studies have focused on the role of Crk proteins as an adaptor protein in the cytoplasm, other work has identified CrkII in the nucleus. For instance, Miller and colleagues examined the over-expression of CrkI/II in lung adenocarcinomas and found equal levels in both the nucleus and cytoplasm using two different antibodies targeting CrkI/II or specifically CrkII (7). Furthermore, we have observed both endogenous and exogenous CrkII staining in both the nucleus and cytoplasm through immunofluorescence on various tumor cell lines (data not shown). A recent study has shown CrkII in both the cytoplasm and the nucleus, although CrkII expression in the nucleus was less than 10% (20). Several CrkII SH3N binding partners, such as DOCK180 and Abl were also found to be associated with CrkII in the nucleus (20). Although this study identified CrkII as a mediator of apoptosis when targeted to the nucleus, other functions of CrkII in the nucleus can not be excluded. TUNEL staining in the virgin mammary glands did not reveal significant apoptosis, thus, perhaps the role of CrkII in the nucleus could depend upon which SH3 proteins it is bound to. Furthermore, CrkL has been found to play an active role in the nucleus, acting as a transcription factor within many different cell types, however, few studies have examined the role of CrkII in this context (21-24). It would be interesting to assess how the localization of CrkII in the mammary epithelium affects mammary gland development and tumorigenesis. Overall, the CrkII transgene is well expressed in a manner consistent with other over-expression studies.

Normally, the majority of mammary gland development initiates during the onset of puberty. Beginning at three weeks of age, the terminal end buds form and begin the process of ductal elongation until they have reached the edge of the fat pad, upon which they then regress (15). MMTV-CrkII mice exhibited a developmental abnormality such that the ductal outgrowth was delayed as early as 5 weeks of age and was not complete at 12 weeks of age, when the mammary fat pad is normally filled with epithelial ducts. This retarded phenotype is similar to other mouse models, such as MMTV-heregulin, MMTV-EphB4, MMTV-Cripto and Krct mice (19, 25-27). Delayed outgrowth appears to be the result of increased collagen surrounding the terminal end buds, especially since no significant changes in cellular proliferation, apoptosis, and differentiation were found (Supplemental data). The increase in collagen implies that the over-expression of CrkII results in an altered stromal-epithelial interaction during terminal end bud outgrowth, and hence a slowed development. However, whether CrkII induces the formation of collagen or inhibits its breakdown remains to be determined.

Through in-vitro studies, CrkII has shown to play a pivotal role in controlling the ability of cells to migrate or invade, however its role in-vivo is less understood (28). For instance, over-expression of CrkII and its binding partner, p130Cas was associated with increased survival and invasion in a 3D collagen assay (29). However, EphrinB2 can inhibit the ability of CrkII to promote migration and invasion through up-regulation of its negative regulator Abl (30). Expression of the EphrinB2 receptor, EphB4 is confined to proliferative phases of mammary gland development, such as puberty and the estrous cycle (19). It is plausible that expression of EphrinB2 and EphB4 prevents CrkII over-expressing epithelial cells from invading the surrounding collagen. Since CrkII is

downstream from a variety of signals involved in puberty (ie. EGFR, Met receptor tyrosine kinase (RTK), integrins, EphB4 RTK), further study is warranted to determine how CrkII results in a delay in ductal outgrowth (19, 31-33).

Although over-expression of CrkII results in the delayed outgrowth of the mammary epithelia, the ductal tree eventually fills the fat pad at later time points (ie. 14 weeks). MMTV-CrkII transgenic glands also display enhanced side branching at 20 weeks of age, which is associated with increased proliferation. We confirmed that altered branching patterns in MMTV-CrkII mice were not due to changes in the estrous cycle (data not shown). Consistently, mice over-expressing p130Cas, a major binding partner of Crk, also displays a similar phenotype (34). Furthermore, other adaptor proteins, such as Shc and Grb2 also show a comparable phenotype when driven by the MMTV promoter, suggesting that these adaptor proteins may potentiate the action of specific growth factors during normal mammary gland development (35). CrkII is downstream from several growth factors that have been shown to induce branching morphogenesis, either in-vitro or in-vivo, such as EGF, HGF, IGF, and TGF- $\beta$  (31) (ref). We were unable to dissect a specific signaling pathway significantly over-activated in the MMTV-CrkII mice at 20 weeks of age when compared to wildtype littermates, however alterations in signals may have been turned on at an earlier stage. Regardless, it is clear that over-expression of CrkII alters the normal epithelia development of the mammary gland.

Although mammary epithelial expression of CrkII was capable of altering normal mammary gland development, the incidence of tumors was low. Tumor development was found in both virgin and multiparous females, with a combined tumor incidence of x% and a tumor latency of x days. Although the long latency and low incidence suggests that

other genetic events are required for tumor onset, these results are important, as many other adaptor and scaffold proteins when over-expressed in the mammary gland were unable to induce tumorigenesis. For instance, over-expression of p130Cas, Grb2 and Gab2 were unable to induce tumor formation (34-36). Consistent with the CrkII data, mice over-expressing Shc also have a low tumor incidence of 7% (35). The low tumor incidence of CrkII transgenic mice may be linked to its linker region, containing the Y221 negative regulatory site and its SH3 domain at the C-terminus (37). Phosphorylation of Y221 by binding partners, such as Abl results in the auto-inhibitory structure of CrkII preventing binding from any SH3 binding partners (28, 38). It would be of interest to cross these CrkII transgenic mice with other MMTV-models in which CrkII is a major downstream signaling target.

One CrkII tumor has been identified as a fibroadenoma, which contains regions of epithelial cells (CK8+), mesenchymal cells (Vimentin) and myoepithelial cells (smooth muscle actin, CK14+). It should be noted that expression of Crk was moderate in the fibroadenoma, although this may be due to the activation of a genetic program that is incompatible with the epithelial-specific MMTV promoter, which was believed to occur in tumors from MMTV-ILK mice (17).

Another CrkII tumor was identified as a squamous adenocarcinoma, with a high mitotic index and the presence of cytokeratin pearls. Quantitative real-time PCR specific for the transgene confirmed that this tumor is indeed positive for the Crk transgene. The pathology of the CrkII tumor is similar to MMTV models in which the Wnt pathway is over-expressed, suggesting that it may be a basal-like phenotype, which is often associated with poor prognosis in human cases (44, 45). Basal-like tumors are defined as

subsets of breast cancer that express genes characteristic of basal epithelial cells (ref). Indeed, staining in the CrkII adenosquamous carcinoma was positive for CK14+ (myoepithelial marker), CK8 (epithelial marker) as well as CK6+ (a putative progenitor marker) suggesting that this tumor is indeed basal-like and is of a mixed lineage. Consistent with the MMTV-Wnt mice, there were two predominant cellular components – luminal (CK8+) and myoepithelial (CK14+) (44), suggesting that transformation occurs from a single precursor cell. Although no link has been shown between CrkII and Wnt, it would be interesting to determine if CrkII is expressed in mammary epithelial precursors.

As with the normal mammary epithelium, CrkII was localized in the nucleus or the cytoplasm. In the fibroadenoma, CrkII was mainly cytoplasmic, whereas in the adenosquamous carcinoma, CrkII was mainly nuclear. Future work from this study is to examine the differences between nuclear and cytoplasmic localization of CrkII and its role in mammary tumorigenesis.

In conclusion, over-expression of CrkII is implicated in altering mammary gland development and accelerating tumor development in the presence of other genetic events. Elevated levels of CrkII are found in both luminal and basal human breast cancer, suggesting that the CrkII adaptor protein may be a valuable target for these types of breast tumors.

## **Materials and Methods**

### **Plasmid Construction and Generation of Transgenic Mice**

The CrkII plasmid pSport6-CrkII (Open Biosystems, NM\_016823) was used to generate transgenic mice. The construct was excised using NheI and StuI and then cloned into pDONR201 using the Gateway Vector Conversion System (Invitrogen, Burlington,

Ontario). The pDONR201-CrkII construct was subsequently cloned into an MMTV vector (p206) containing attR cloning sites, the MMTV promoter and a polyadenylation sequence. The MMTV-CrkII-SV40 construct was purified, excised using SpeI and SphI restriction enzymes and sequence verified. To generate transgenic mice, a purified 6.3 Kb MMTV-CrkII fragment was microinjected into pronuclei of FVB and the generation of chimeric mice was carried out at the McGill Transgenic Facility, McGill University. Mice were housed in the Royal Victoria Hospital (Montreal, Canada) transgenic mouse facility and all experiments were carried out in accordance with McGill University Animal Ethics Committee guidelines.

### **Genotyping of Transgenic Mice**

Transgenic mice were identified by PCR using DNA extracted from tail tips (Ref). PCR reactions were performed using two SV40 specific primers: forward 5' GGAACCTTACTTCTGTGGTGT3' and reverse 5'GCATCCCACCACTGCTCCCATTC 3' which gave rise to a product of 350 bp. To examine expression levels of the transgene, total RNA was extracted, isolated and purified from mammary glands using Trizol reagent (Invitrogen, Burlington, Ontario) according to the manufacturer's protocol. The RNA was then DNase treated (Roche, Mannheim, Germany) and re-precipitated in phenol:chloroform:isoamyl alcohol as previously reported (ref). Reverse transcription reactions were carried out using oligodT for priming (Invitrogen, Burlington, Ontario) and SuperscriptII reverse transcriptase (Invitrogen, Burlington, Ontario). Real-time PCR was carried out using the Rotor Gene Real-time thermocycler (Corbett Research, Sydney, Australia). The PCR reaction mixture consisted of cDNA template, 1 uM of each primer, and 5 ul of SYBR Green Master Mix (Qiagen, Mississauga, Ontario). Samples were



amplified with a 15 minute pre-incubation at 95°C, followed by 40 cycles of 94°C for 20s, 55°C for 15s and 72°C for 20s. The housekeeping genes used were ALAS1 and/or GAPDH. ALAS1 primers were 5' CCACTGGAAGAGCTGTGTGA 3' and 3' TGGCAATGTATCCTCCAACA and had a similar PCR program, except the annealing temperature was 53°C. GAPDH primers were 5' TCATGACCACAGTGGATGCC and 3' GGAGTTGCTGTTGAAGTCGC 5', and were amplified with the following changes: an annealing temperature of 61°C for 20s and an elongation temperature of 72°C for 30s.

The standard curve to quantify mRNA expression was established by amplifying serial dilutions of one sample (1, 1:10, 1:100, 1:1000, 1:10000) for each primer set. The values for each sample obtained from the standard curve were then used to determine the expression levels of the transgene by dividing the SV40 value over the housekeeping gene.

### **Wholemout Analysis of Mammary Glands**

Right inguinal mammary glands (#4) were resected, flattened and fixed in acetone (Fisher Scientific) before staining with Mayer's modified hematoxylin (Fisher Scientific, Ottawa, Ontario). The glands were destained with acidified ethanol (1% HCl in 70% ethanol) and then treated sequentially with 0.2% ammonium hydroxide, 70% ethanol, 100% ethanol and xylene before being mounted in permount (Fisher Scientific, Ottawa, Ontario). Wholemount images were captured using AxioVision software (Carl Zeiss, Toronto, Ontario) and a Zeiss microscope (Carl Zeiss, Toronto, Canada).

### **Quantification of Ductal Development from Wholemounts**

To quantitate ductal outgrowth, wholemounts from mice at 12 weeks of age were captured using the microscope and AxioVision software (Carl Zeiss, Toronto, Ontario) at

a magnification of 0.6x. Using Scion Image-NIH equivalent software for Microsoft Windows (Scion Company, Frederick, Maryland), the distance from the lymph node to the terminal end bud and the lymph node to the edge of the fat pad was calculated. The value of the terminal end bud over the total fat pad was determined and expressed as a percentage.

### **Histology**

Left inguinal mammary glands (#4) were fixed in 10% neutral buffered formalin (Surgipath, Richmond, IL) overnight at 4°C. The glands were then washed 1x with PBS and transferred to 70% ethanol and paraffin embedded. Sections were stained with hemotoxylin and eosin, or Masson's Trichrome staining.

### **Immunohistochemistry**

All paraffin sections (10 µm) were deparaffinized and rehydrated in a series of incubations in xylene and 100%, 95%, 80% and 70% ethanols. Sections were subsequently washed in 1x PBS. All antibodies and blocking reagents were diluted in PBS. Antigen retrieval was performed using 10 mM sodium citrate pH 6.0 and then sections were washed 4x in water for 3 minutes each followed by one 3 minute wash with PBS. Endogenous peroxidases were removed by washing in 3% hydrogen peroxide (Fisher Scientific, Ottawa, Ontario) for 15 minutes. Sections were then washed with PBS. Blocking was carried out for 60 minutes using 2% BSA. After blocking, the sections were covered with primary antibody and incubated overnight at room temperature. After washing, the secondary antibody was added for ½ hour at room temperature. Sections were subsequently washed and if biotinylated, subjected to an ABC reagent for 30 minutes (Vector, Burlingame, CA, USA). After washing, sections underwent a DAB

substrate reaction (DAKO Cytomation, Glostrup, Denmark), were counterstained with hematoxylin and mounted using Acyrol. Primary antibodies included: Crk (1:200), CK8 (1:1200), CK6 (1:800), CK14 (1:1000), smooth muscle actin (1:500), PCNA (1:1500), vimentin (1:1500). Staining for PCNA was carried out using the Mouse on Mouse (MOM) staining kit, according to the manufacturer protocol (Chemicon, Temecula, California). The secondary antibody for Crk and smooth muscle actin was anti-mouse HRP (1:500), whereas CK14 and CK6 used the anti-rabbit Vectastain biotinylated antibody (Vector, Burlingame, CA, USA). Finally, CK8 used the biotinylated anti-guinea pig and vimentin required the biotinylated anti-goat secondary antibody (Vector, Burlingame, CA, USA). The PCNA antibody was purchased from DAKO Cytomation (Glostrup, Denmark), Cytokeratin 8/18 was from Fitzgerald Industries International (Concord, MA), and Cytokeratin 14 and Cytokeratin 6 were purchased from Covance (Berkley, CA). Monoclonal antibodies for Crk, vimentin and smooth muscle actin were purchased from BD Transduction Laboratories (Lexington, KY) and Sigma Aldrich (Oakville, Ontario) respectively.

### **Protein Analysis and Antibodies**

Mouse mammary glands were lysed in 1% Triton X-100 lysis buffer containing 50mM HEPES pH7.5, 150mM NaCl, 2mM EGTA, 1.5mM MgCl<sub>2</sub>, 1mM PMSF, 1mM Na<sub>3</sub>VO<sub>4</sub>, 50mM NaF, 10µg/ml aprotinin and 10µg/ml leupeptin. Whole cell lysates were resolved by SDS-PAGE in 12% gels. Western blot was performed as previously described (18) by transferring the separated proteins onto a Hybond-ECL nitrocellulose membrane from Amersham Biosciences (Baie d'Urfe, Canada). The monoclonal antibody for Crk was purchased from BD Transduction Laboratories (Lexington, KY) whereas

actin was purchased from Santa Cruz (Santa Cruz, California).

### **Analysis of Tumor Formation**

Mammary glands with visible tumor formation were resected. Tumor pieces were embedded in OCT or snap frozen in liquid nitrogen or fixed in 10% neutral buffered formalin.

### **Statistical Analysis**

Results were expressed as the mean +/- standard error. Statistical analysis of data was done using a Student's t test.

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## Figure Legends

**Figure 1:** Expression of the CrkII transgene in the mammary epithelium. (A) The design of the MMTV-CrkII transgene. (B) Expression of CrkII protein in transgenic and FVB mice in both virgin (V) and lactating (L) animals (6394-6398 indicate the various transgenic mouse lines). Although the antibody recognizes endogenous protein, it is evident in lactating (and some virgin) animals that CrkII is higher in transgenic mice than wildtype FVB mice. (C) Expression of transgene using primers specific for the SV40 region indicates the CrkII transgene is well expressed in each transgenic line. House keeping genes examined include GAPDH or ALAS1. Semi-quantitative real-time PCR data illustrating the varying expression levels of the transgene in virgin (V) and lactating (L) female mice amongst the various transgenic lines (D). Immunohistochemistry of CrkII (ii, iii) in virgin transgenic mouse mammary epithelium compared to a normal FVB littermate (i). Images i and ii taken at 20x, iii taken at 40x. Scale bar represents 50  $\mu$ m.

**Figure 2:** Delayed ductal outgrowth in MMTV-CrkII transgenic mice compared to normal FVB littermates reveals an increase in stroma surrounding the terminal end bud. (A) Delayed ductal outgrowth in transgenic mice was detected at 5 weeks (data not shown), 10 weeks (ii) and 12 weeks (iv) relative to FVB mice at the same age, (i) and (iii) respectively. Images taken at 0.6x. (B) The percentage of fat pad filled for each of the 5 MMTV-CrkII mouse lines at 12 weeks of age relative to an FVB control (i). The percentage of fat pad filled by epithelial ducts was calculated using Scion Image for 6 FVB mice and 6 CrkII mice at 10 weeks of age and the average percentage was determined  $\pm$  standard error (ii) (C). Comparison of FVB terminal end buds (i) to those



from MMTV-CrkII transgenic mice (ii) implies that CrkII terminal end buds have increased stroma and components of the basement membrane encompassing the end bud. Trichrome staining revealed that increased collagen surrounds the MMTV-CrkII transgenic end bud structures (iv) but not wild-type terminal end buds (iii). Immunohistochemistry reveals that Crk is indeed expressed in the terminal end buds of transgenic mice (vi) but not in FVB littermates (v). All images taken at 20x. Scale bar represents 50  $\mu$ m.

**Figure 3:** Enhanced branching of MMTV-CrkII mice at 20 weeks of age. (A) In comparison to a wildtype FVB littermate (i, 2x), MMTV-CrkII transgenic mice show enhanced branching (ii, 2x). Branching ducts express high levels of CrkII (iv) relative to normal FVB littermates (iii). Staining for PCNA reveals active proliferation of the epithelial cells in MMTV-CrkII mammary ducts (vi) whereas little to no proliferation is visualized in FVB mice (v). All immunohistochemistry images taken at 20x. Scale bar represents 50  $\mu$ m. (B) Aging virgin CrkII mice show a wide variety of phenotypes. Aging virgin mice sacrificed between 12-15 months display atypical epithelial architecture, such as lobuloaveoli (ii, 6.6x), spiculated ends (iii, 6.6x) and hyperplasia (iv, 6.6x) relative to an FVB mouse of the same age (i, 0.6x).

**Figure 4:** MMTV-CrkII over-expression leads to tumor development. (A) A adenosquamous carcinoma was found in a multiparous CrkII mouse at 397 days and expresses the Crk protein (ii), CK14 (iii), CK8 (iv) and CK6 (vi). The tumor was highly proliferative, as illustrated by PCNA staining (v). (B) A focal fibroadenoma in an aging

virgin mouse at 446 days of age (i) expressed Crk (ii), CK14 (iii), CK8 (iv), smooth muscle actin (v) and vimentin (vi). All images taken at 20x where the scale bar represents 50  $\mu\text{m}$ .

**Table 1:** MMTV-CrkII tumor incidence. Mice from two MMTV-CrkII transgenic lines were set up into 2 groups - virgin or multiparous and monitored for tumor development. Preliminary results show that 7 multiparous females from line 6396 and 6 multiparous females from line 6398 as well as and 7 virgin mice (4 from 6396, 3 from 6398) have each developed tumors. Each line (6396 and 6398) has a tumor incidence of 34% and 36% respectively, with a combined rate of 35%. Age-matched FVB developed tumors, with an incidence of 18%.

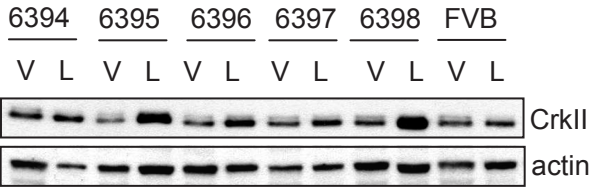
**Supplemental Figure 1** - Delayed ductal outgrowth in MMTV-CrkII transgenic mice compared to normal FVB littermates reveals no significant changes in proliferation, apoptosis or differentiation of the terminal end bud. Paraffin embedded sections of 10 week old mammary glands from 6 FVB and 6 MMTV-CrkII mice were stained for PCNA, revealing that terminal end bud structures are proliferative in both cohorts of mice (i, ii). Further comparison of FVB terminal end buds (iii) to those from MMTV-CrkII transgenic mice (iv) implies that overall apoptosis levels are similar, using the ApopTag Plus Peroxidase In Situ Detection system. No significant changes were also observed in staining patterns of CK8 (v, vi) or CK14 (vii, viii) in FVB (v, vii) versus CrkII (vi, viii) transgenic mice. All images taken at 20x where the scale bar represents 50  $\mu\text{m}$ .

**Supplemental Figure 2** – Over-expression of CrkII does not significantly alter mammary gland development during lactation, pregnancy or involution. Analysis of H&E stained paraffin embedded sections of mice at lactation day 15, pregnancy day 10 and day 18, involution day 3 and day 20 reveals no significant alterations in the mammary epithelium between wildtype and CrkII transgenic mice. All images taken at 20x. Scale bar represents 50  $\mu$ m.

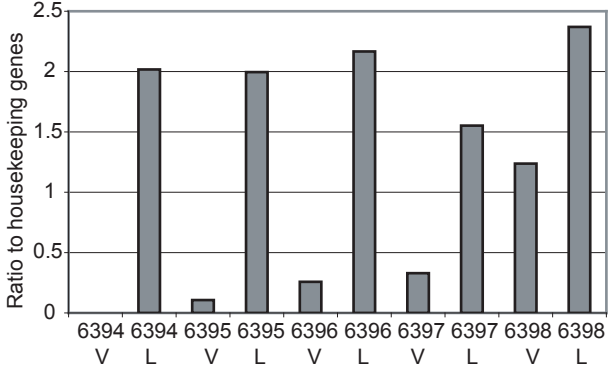
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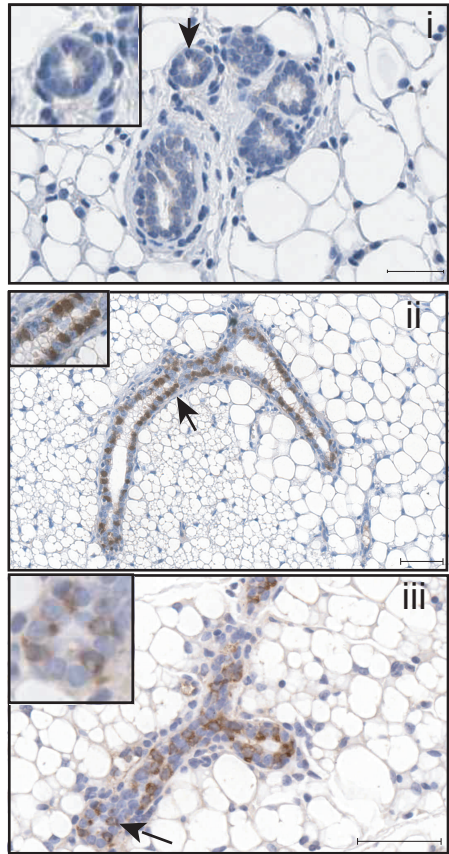
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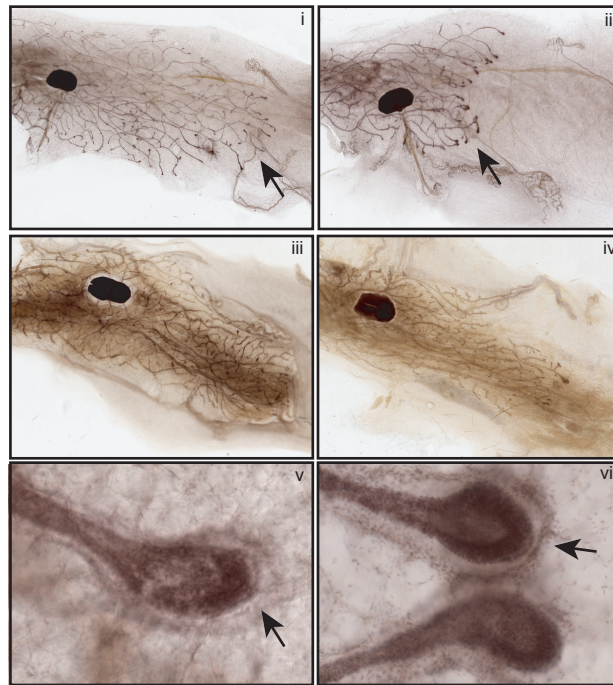
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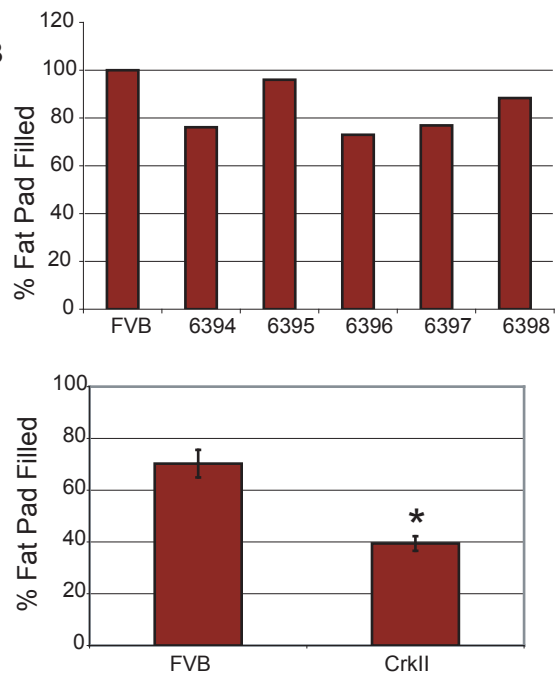
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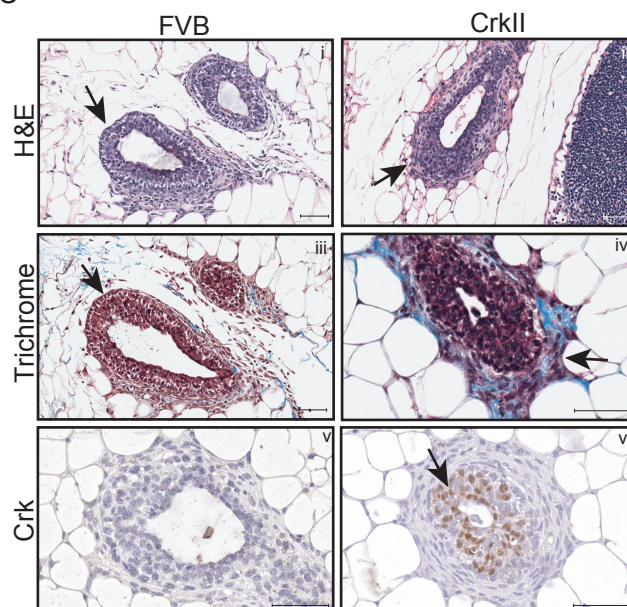
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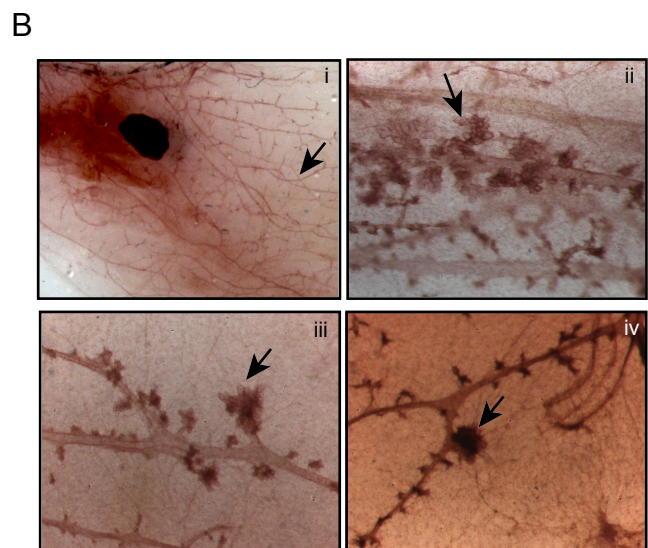
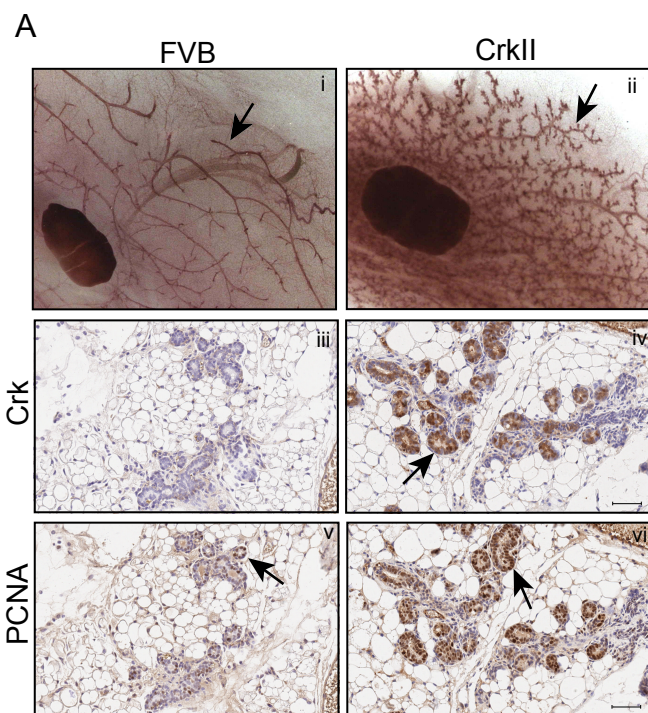


B



C





Line	# of glands analyzed		# of glands with hyperplasias/tumors		Incidence per line	Combined incidence	Latency
	Virgin	Multiparous	Virgin	Multiparous			
6396	16	16	4	7	34.4	35.2	470
6398	12	13	3	6	36.0		452
FVB	15	14	0	6	18.7	18.7	464



